

BIOLOGICAL ACTIVITIES OF A HUMAN PLURIPOTENT HEMOPOIETIC COLONY STIMULATING FACTOR ON NORMAL AND LEUKEMIC CELLS

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Colony stimulating factors (CSF)¹ are a class of hemopoietic regulatory glycoproteins required for survival, proliferation, and differentiation of hemopoietic stem and progenitor cells in vitro, and probably in vivo (1, 2). Many efforts have been undertaken to characterize the biochemical and biological properties of these factors. In the murine system, CSF stimulating the growth of progenitor cells of neutrophil granulocytes (G-CSF), of macrophages (M-CSF or CSF-1), of macrophages and granulocytes (GM-CSF), and of mixed colonies containing erythrocytes, granulocytes, macrophages, and megakaryocytes (multi-CSF or interleukin 3 [IL-3]) have been purified to homogeneity (3-6). It is not yet known whether these four murine CSF account for all biological activities originally ascribed to a greater number of specialized colony-stimulating activities that had been defined operationally on the basis of colony morphology. Through the advent of molecular biology, it has become possible to genetically clone some of these factors (7, 8).

Initial attempts to purify human CSF were less successful. Recently however, CSF-1, GM-CSF, and erythroid potentiating activity (EPA), a CSF-like activity, have been purified to homogeneity (9-12), and human GM-CSF has been genetically cloned and sequenced (13).

We have purified to apparent homogeneity a pluripotent hemopoietic colony stimulating factor from conditioned media of the human urinary bladder carcinoma cell line, 5637 (14). The purified molecule, which we have termed pluripotent CSF (pCSF) or pluripoietin, supports colony formation of normal human bone marrow in vitro from multipotential (colony forming unit of granulocytes/erythrocytes/macrophages/megakaryocytes [CFU-GEMM]) and early erythroid (burst forming unit of erythroid cells [BFU-E]) progenitors, as

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¹ *Abbreviations used in this paper:* ANAE, α -naphthyl acetate esterase; BFU-E, burst-forming unit of erythroid cells; CFU-GM, CFU of granulocyte/macrophage; CM, conditioned medium; CML, chronic myelogenous leukemia; CSF, colony-stimulating factor; DF, differentiating factor; EPA, erythroid potentiating activity; FBS, fetal bovine serum; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; IL-3, interleukin 3; LAL, limulus amoebocyte lysate; LFB, Luxol Fast Blue; PBS, phosphate-buffered saline; pCSF, human pluripotent colony stimulating factor (pluripoietin); RP-HPLC, reverse-phase high-performance liquid chromatography; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TdR, thymidine.

well as unipotential and bipotential granulocyte and macrophage progenitor cells (CFU-GM). IL-3, the only murine CSF known to support multipotential colony growth, has a variety of biological activities (reviewed in 15, 16) on hemopoietic progenitor cells, mast cells, and their precursors, and possibly on lymphocytes (17). We wished to study additional biological effects of purified pCSF on progenitors and mature cells of normal and malignant hemopoiesis in mouse and man.

Materials and Methods

Cell Lines. The human leukemia cell line, KG-1 (passage 21) and KG-1a, a line with an undifferentiated blast-like appearance, which was spontaneously derived from KG-1 (18), were generously provided by H. Koeffler, Department of Medicine, University of California, Los Angeles. The HL-60 promyelocytic leukemia cell line, originally obtained from R. Gallo (National Cancer Institute, Bethesda, MD), and WEHI-3B (D+) murine myelomonocytic cell line were continuously maintained in our laboratory. Cell lines were tested for consistency with published characteristics using morphologic, cytochemical, and karyotypic analysis (19). The murine IL-3-dependent cell line, FDC-P2 was obtained from T. Dexter (Department of Experimental Hematology, Paterson Laboratories, Wilmington, Manchester, United Kingdom).

Separation of Bone Marrow Cells. Bone marrow cells from healthy human volunteers, who gave informed consent, were separated and depleted of adherent cells and T lymphocytes, as described (20, 21). Briefly, after density gradient centrifugation on Ficoll-Hypaque, and washing, low-density cells were depleted of adherent cells by incubation on plastic surfaces in RPMI 1640 supplemented with glutamine, antibiotics, and 10% heat-inactivated fetal bovine serum (FBS). Nonadherent cells were recovered by gently rinsing the dishes with medium, and were further depleted of T lymphocytes by rosetting with neuraminidase-treated sheep red blood cells, and by density gradient separation on Ficoll. The final cell population contained <2% monocytes, as judged using α -naphthyl acetate esterase (ANAE) stain, and <1% T lymphocytes by E-rosetting. Fresh leukemic marrow cells from a patient with newly diagnosed Ph¹-positive chronic myeloid leukemia (CML) in chronic phase were separated by density gradient centrifugation as described above. This population of cells contained <5% ANAE-positive cells, and >90% had the morphological appearance of myeloid cells and their precursors after Giemsa staining.

CFU-GM Colony Assay and Pre-CFU Assay. For CFU-GM assay, low density, nonadherent, and T cell-depleted normal human bone marrow cells were cultured in 0.3% agar in supplemented McCoy's medium, as described (22). When cultured at concentrations of up to 10^5 cells/ml for 7–14 d at 37°C in 5% CO₂ in air at 100% humidity, no colony (aggregates of >40 cells) or cluster (1–40 cells) formation was observed without the addition of exogenous sources of CSF. Therefore, this cell population seemed largely deprived of endogenous producers of CSF. For study of progenitor cells of CFU-GM, termed pre-CFU (23), the same cell population was incubated in liquid culture for 5, 7, or 9 d before agar culture. Quadruplicate cultures of 2.5×10^4 cells in 100 μ l/well were incubated in 96-well flat-bottom tissue culture trays in Iscove's modified Dulbecco's medium (Gibco Laboratories, Grand Island, NY) supplemented with 30% FBS, 5×10^{-5} M 2-mercaptoethanol, and serial dilutions of purified pluripotent CSF or control medium at 37°C in 5% CO₂ in air at 100% relative humidity. Contents of each well were then resuspended and incorporated into a 1-ml system in supplemented McCoy's, and cultured as outlined above for the CFU-GM assay. Because the purpose of this experiment was to determine the ability of pCSF to support survival and growth of pre-CFU in liquid culture, we used as an external source of CSF for the final culture, in agar-saturating concentrations (10% vol/vol) of human bladder carcinoma cell line 5637-conditioned medium (CM). This CM contains other growth factor(s) in addition to pCSF, and it supported optimal colony growth from CFU-GM progenitor cells surviving or generated during suspension culture with purified pCSF alone. For morphological studies of colonies, whole agar dishes

from CFU-GM cultures were transferred to glass slides and stained for ANAE, Luxol Fast Blue (LFB) or Kaplow's myeloperoxidase with Harris' hematoxylin counterstain (21).

Assay for Leukemia Differentiation Induction. Titrated samples of purified pCSF, or dialyzed reverse-phase high-performance liquid chromatography (RP-HPLC) fractions were tested for differentiation induction (GM-DF [differentiating factor] activity) of WEHI-3B (D+) or HL-60 leukemia cells in a clonal assay system (24, 25). Briefly, 3×10^2 cells/ml WEHI-3B (D+) or 10^3 cells/ml HL-60 cells were incubated in 0.3% agar in supplemented McCoy's medium containing 12.5% FBS in 0.2 ml/well in 24-well tissue culture trays (Costar, Cambridge, MA) in replicates at 37° in 5% CO₂ in air. Cultures were scored on day 7 (WEHI-3B) or day 14 (HL-60) for induction of disperse, differentiated colonies or tight, blast cell colonies. Total cloning efficiency was close to 30% for WEHI-3B (D+) and 10% for HL-60 in all assays, and was not changed significantly in the presence of pCSF. This assay allows for easy quantitation of GM-DF activity, and closely parallels differentiating activity as measured by morphological and functional criteria (26, 27). Analogous to the definition of units (U) of GM-CSF activity, 50 U of GM-DF activity were arbitrarily defined as inducing 50% differentiated colonies. For morphological analysis of leukemic cell colonies, whole agar plates from 1-ml cultures in petri dishes were stained as described above.

Assay for [³H]Thymidine Uptake. Leukemic cell lines or bone marrow cells from a patient with leukemia (see above) were cultured at different cell concentrations in supplemented RPMI 1640 containing 10% FBS, with varying concentrations of purified pCSF or control medium in 96-well flat-bottom plates (3596; Costar) at 37°C in 5% CO₂ in air. After the indicated periods of time, cultures were pulsed for 4 h with 0.5 μCi/well [³H]thymidine ([³H]TdR) (New England Nuclear, Boston, MA), and the [³H]TdR uptake measured as described previously (28).

Assay for Binding of Chemotactic Peptide. Mature granulocytes were obtained from peripheral blood of healthy human volunteers by buoyant density centrifugation. Briefly, buffy-coat cells from heparinized peripheral blood were diluted 1:3 with phosphate buffered saline (PBS), layered over a discontinuous gradient of Ficoll-Hypaque of densities 1.077 and 1.119 (Sigma Chemical Co., St. Louis, MO), and centrifuged at 2,000 rpm for 40 min at room temperature. Cells from the lower interphase (1.119) were harvested, washed three times, and adjusted to a concentration of 10^6 viable cells per milliliter of RPMI 1640 containing 10% FBS. This cell preparation contained >95% polymorphonuclear cells by Giemsa stain. For induction of receptors for *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), cells were incubated at the given concentration in a total volume of 1 ml, in the presence or absence of purified pCSF at 37°C for varying periods of time. Measurement of specific binding of fMLP was performed as described, with minor modifications (29). Shortly, supernatant was removed after centrifugation, and cells were incubated with 15 nM [³H]fMLP (New England Nuclear) in 150 μl medium for 3 h at 4°C. Radioactivity associated with cells was determined by harvesting cells onto glass fiber disks, washing with cold PBS, and counting by liquid scintillation spectroscopy. Specific binding was calculated as the difference of activity (cpm) bound in the absence and presence of 100 μM cold fMLP.

Biochemical Procedures. Biochemical purification and separation of pCSF was performed using sequential ammonium sulfate precipitation, anion-exchange chromatography, gel filtration, and RP-HPLC, as described (14). For the experiment described here, fractions containing pCSF from anion-exchange column and gel filtration were loaded onto a μBondapak C₁₈ column in 0.9 M acetic acid/0.2 M pyridine buffer, pH 4.0, with 20% 1-propanol, and subsequently eluted with a linear gradient of 20–50% 1-propanol in acetic acid/pyridine buffer at a flow rate of 1 ml/min. Purified pCSF migrated as a single band of *M*_r 18,000 in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration of the band was estimated by comparison with serial dilutions of known amounts of proteins in SDS-PAGE.

Assay for Bacterial Endotoxins. The preparations of purified pluripoietin (pCSF) used in these studies were tested for endotoxin contamination in limulus amoebocyte lysate (LAL) assays, kindly performed by R. Sholman (New York Blood Center), or using an

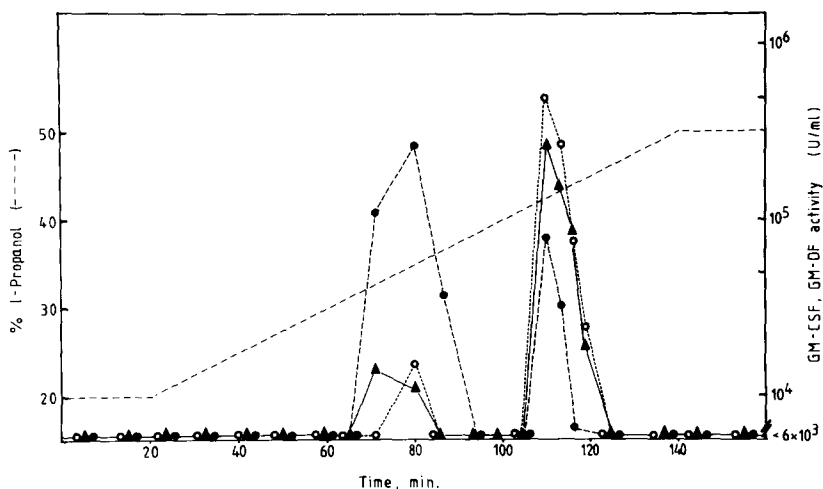


FIGURE 1. Pooled fractions with pluripotent activity eluted from a gel filtration column were acidified and loaded onto a RP-HPLC C 18 column in 20% 1-propanol (see Materials and Methods). Bound proteins were eluted with a linear gradient of 20–50% 1-propanol. Fractions of 3 ml each were collected and tested for pluripoiectin (\blacktriangle) and GM-DF activity on HL-60 (\bullet) and WEHI-3B (D+) (\circ) leukemic cells.

FDA-approved LAL test kit (Byk Mallinckrodt, Konstanz, Federal Republic of Germany). Results obtained with different batches of pCSF indicated endotoxin contents ranging from $<1 \text{ ng}/10^6 \text{ U}$ of pCSF to $<1 \text{ ng}/1.9 \times 10^4 \text{ U}$ of pCSF.

Results

Pluripoiectin Induces Differentiation of Leukemia Cell Lines. Pluripotent CSF from CM of human bladder carcinoma cell line 5637 was purified by sequential ammonia sulfate precipitation, anion-exchange chromatography, and gel filtration, as described previously (14). In the final purification step on RP-HPLC, pCSF measured as GM-CSF and GM-DF for both HL-60 and WEHI-3B (D+) eluted together at a 1-propanol concentration of 42% (Fig. 1). pCSF from this fraction appeared to be purified to homogeneity (14); it migrated as a single band in silver-stained SDS-PAGE coincident with GM-CSF activity and GM-DF on both leukemia cell lines (data not shown).

An additional peak of GM-DF activity eluted at a lower 1-propanol concentration, separate from pCSF (Fig. 1). Fractions from this peak were highly active in inducing differentiation of HL-60 cells, but had low activity on WEHI-3B (D+) cells and in the GM-CSF assay on day 7. Purification of the material contained in this peak, designated pluripoiectin- α , will be published elsewhere.²

HL-60 and WEHI-3B (D+) leukemia cells were differentially sensitive to differentiation induction by pCSF. Two independent purification procedures resulted in virtually identical specific biological activities of $1.25\text{--}1.5 \times 10^8 \text{ U GM-CSF/mg protein}$, and in very similar GM-CSF/GM-DF activity ratios (Table I). WEHI-3B (D+) cells were clearly more sensitive to differentiation induction

² Gabrilove, J. L., K. Welte, P. Harris, E. Platzer, L. Lu, R. Mertelsmann, and M. A. S. Moore. Manuscript submitted for publication.

TABLE I
Colony-stimulating and Leukemia-differentiating Activity of Purified Pluripoietin

Purification	GM-CSF activity		GM-DF activity WEHI-3B (D+)		GM-DF activity HL-60	
	Specific activity ($\times 10^8$ U/mg protein)	U/ml $\times 10^3$	U/ml $\times 10^3$	DF/CSF ratio	U/ml $\times 10^3$	DF/CSF ratio
I	1.5	84	246	2.9	54	0.6
II	1.25	201	502	2.5	80	0.4

Purified pluripoietin from two independent purifications (I and II) is compared for its ability to support CFU-GM (GM-CSF activity), and to induce differentiation in human HL-60 and murine WEHI-3B (D+) leukemia cells (GM-DF activity) in clonal assays. Units (U) of activity are defined in Materials and Methods. Due to different amounts of starting material, the concentrations of biological activities differ between I and II, but are similar relative to each other.

by pCSF than was HL-60, which required approximately five times more pCSF for comparable effects.

Morphologically and cytochemical analysis of HL-60 colonies was performed using ANAE and LFB stains as markers of the monocyte/macrophage and eosinophil granulocyte lineage, respectively, with Harris' hematoxylin counterstain for nuclear morphology. In the presence of pCSF we observed an increase (of up to 75%) in the percentage of HL-60 colonies containing a significant number (>10%) of polymorphonuclear cell colonies, as compared to 15–20% for control medium, and the appearance of some LFB⁺ cells, as well as an increase in intensity of ANAE stain. WEHI-3B (D+) colonies, although positive for ANAE when grown in the absence of pCSF, stained with higher intensity for ANAE when induced with pCSF. Taken together, these data suggest that pCSF may induce differentiation of leukemia cells towards granulocytes and/or macrophages (GM-DF).

Pluripoietin Induces [³H]TdR Uptake in Fresh Leukemia Cells and in KG-1 Leukemia Cell Line. Light-density bone marrow cells from a previously untreated patient with Ph¹-positive CML were incubated with titrated samples of purified pCSF. A dose-dependent increase in [³H]TdR uptake was observed in the presence of pCSF as compared to control medium on day 3 of culture (Table II). Similar changes in [³H]TdR uptake were obtained after 5 and 7 d of culture, and in the 7-d colony assay in semisolid agar using the same cells (data not shown).

KG-1 leukemia cells with myeloblast to promyelocyte morphology are known to be susceptible to growth modulation in vitro by CSF (30). Significant enhancement of [³H]TdR uptake was seen in KG-1 cells (passage 24) in the presence of pCSF within 24 h (Table III). In this particular experiment, pCSF could be detected at 13 U/ml; yet 25–50 U/ml consistently gave significant enhancement of [³H]TdR uptake. Passages later than 45 gradually lost this responsiveness, giving an increase only $\leq 25\%$ in [³H]TdR uptake. A somewhat higher increase of up to twofold over medium control could be seen with early passages of KG-1 after 3 d of incubation. [³H]TdR uptake of KG-1 cells or, as shown above, fresh CML marrow cells may be used as a fast and sensitive screening assay to

TABLE II
 $[^3\text{H}]\text{TdR}$ Uptake by CML Marrow Cells in Response to Pluripoietin

Treatment	$[^3\text{H}]\text{TdR}$ uptake	Stimulation index
	<i>cpm</i>	
Control medium	2,144	1.0
Pluripoietin (U/ml)		
500	17,123	8.0
250	14,384	6.7
125	11,828	5.5
63	6,939	3.2
31	4,505	2.1
15	5,356	2.5
Crude 5637-CM		
500 U/ml	23,912	11.2

Light-density bone marrow cells from a previously untreated patient with Ph^1 + CML in chronic phase were cultured at 2×10^5 cells/200 μl /well with titrated samples of purified pluripoietin, or control medium for 3 d. Results are expressed as mean cpm from triplicate cultures. Stimulation index is the ratio of cpm from cultures in the presence of pCSF to cpm from control cultures.

TABLE III
 $[^3\text{H}]\text{TdR}$ Uptake by Human Leukemic Cell Line KG-1

Treatment	$[^3\text{H}]\text{TdR}$ uptake	
	<i>cpm</i>	%
Control medium	3,660 \pm 108	100
Pluripoietin (U/ml)		
200	5,366 \pm 176	147
100	5,245 \pm 4	143
50	4,935 \pm 180	135
25	4,553 \pm 178	130
13	4,553 \pm 6	124
6	4,363 \pm 493	119

KG-1 cells of passage 24 were incubated at 15,000 cells/well for 24 h with titrated samples of purified pluripoietin, or control medium. Results are expressed as mean cpm \pm SD for triplicate cultures. Differences are significant on a 5% level at ≥ 13 U/ml, using a two-tailed Student's *t*-test.

monitor purification of pCSF and pluripoietin- α . KG-1a, an undifferentiated variant of KG-1, did not respond to pCSF by increased $[^3\text{H}]\text{TdR}$ uptake (data not shown).

In 14-d semisolid agar culture, pCSF enhanced colony formation from KG-1 cells (passage 50) by 1.7–3.1-fold, at a cloning efficiency of 1.5–3.2% at 3×10^3 cells/ml (data not shown). Also, a pronounced increase in colony size was observed.

Pluripoietin Promotes Survival and Proliferation of Normal Hemopoietic Progenitor Cells and Their Precursors. In the CFU-GM assay in semisolid agar, purified

TABLE IV
Activity of Pluripoietin on Pre-CFU

Pluripoietin concentration <i>U/ml</i>	Exp. 1 (7 d in suspension culture)	Exp. 2 (7 d in suspension culture)	Exp. 3	
			5 d in suspension culture	9 d in suspension culture
1,000	260 ± 18	80 ± 11	128 ± 20	320 ± 32
500	307 ± 20	156 ± 16	NT	NT
100	200 ± 18	116 ± 29	292 ± 17	122 ± 21
10	145 ± 15	48 ± 11	203 ± 13	136 ± 18
Control medium	104 ± 20	32 ± 9	105 ± 21	81 ± 13

Light-density, adherent cell and T lymphocyte-depleted normal human bone marrow cells were incubated in suspension culture with titrated samples of purified pluripoietin or control medium. After indicated periods of time, cells were resuspended and plated in semisolid agar culture with saturating concentrations of exogenous CSF (5637-CM), and colonies were counted 7 d later. Results are expressed as mean ± SD colonies from quadruplicates per 10^5 cells input on day 0 of suspension culture. The numbers of colony-forming progenitors input at that time were 204 ± 10 , 104 ± 4 , 88 ± 12 , for Exp. 1, 2, and 3, respectively.

pCSF stimulates colony formation from normal human bone marrow cells. On day 7 of culture, >90% of colonies observed were granulocytic, whereas on day 14, granulocytic, monocytic, and mixed granulocytic/monocytic colonies and some eosinophilic clusters were seen (data not shown). When used at a concentration of 500 U/ml, pCSF also supported human BFU-E and CFU-GEMM colony formation in methylcellulose in the presence of erythropoietin (14). CFU-GEMM possess limited self-renewal and high proliferative capacity *in vitro*, and are probably closely related to hemopoietic stem cells (31, 32). CFU-GM on the other hand, are committed to proliferate and differentiate along the GM pathway, having minimal self-renewal capacity *in vitro*. Since pCSF works on both early and late progenitor cells, we wished to study its activity on survival and generation of CFU-GM from pre-CFU progenitor cells, which are thought to comprise earlier cell types in the hierarchy of hematopoietic stem and progenitor cells (23). Pre-CFU are detected by suspension culture in the presence of CSF before assay for clonal growth in semisolid agar culture. This procedure is assumed for proliferation and terminal differentiation in liquid culture of committed CFU-GM progenitors present at initiation of culture, which cannot subsequently be detected in agar culture. Light-density normal human bone marrow cells, devoid of endogenous producers of CSF by depletion of adherent cells and T lymphocytes, were cultured at low cell densities. As expected, extensive proliferation and differentiation of target cells were observed during suspension culture in the presence of pCSF (data not shown). Purified pCSF supported the survival, expansion and differentiation of early progenitor cells (pre-CFU) to committed CFU-GM progenitors, which then gave rise to day 7 colonies in semisolid agar culture (Table IV). In two of three experiments, the number of CFU-GM progenitors present in liquid culture declined in the absence of pCSF (Table IV), consistent with the requirement for CSF for survival of progenitor cells (33). In one additional experiment (data not shown), we observed an increase in CFU-GM after 7 day suspension culture in the absence of

TABLE V
Induction of Chemotactic Peptide Binding in Mature Granulocytes by Pluripoietin

Treatment time	[³ H]fMLP specific binding
<i>min</i>	<i>cpm</i>
0	1,040 ± 140
10	1,580 ± 210
20	1,900 ± 420
30	3,740 ± 1,250

Highly enriched polymorphonuclear cells from peripheral blood of a normal donor were preincubated with 5,000 U/ml of purified pluripoietin at 37°C for the periods of time indicated, before assay for fMLP binding at 4°C. Specific binding is calculated as the difference of [³H]fMLP binding in the presence or absence of excess cold fMLP. Pooled results from two independent experiments are presented as mean cpm ± SE.

exogenous CSF. Cells from this particular volunteer on several occasions exhibited a very high cloning efficiency in agar cultures, despite the absence of any recognizable pathophysiologic situation. In this experiment, pCSF enhanced the number of CFU-GM surviving or generated in liquid culture.

In all experiments, >90% of colonies grown in the final agar culture contained granulocytes and their precursors.

Pluripoietin Shows Little Cross-species Activity on Murine Cells Other Than WEHI-3B (D+). When normal murine bone marrow cells from CBA/Hn mice were plated in semisolid agar, pCSF supported purely granulocytic colony formation on day 7 at low cloning efficiency. Saturating amounts (500 U/ml) of pCSF supported growth of 8.5 ± 0.7 colonies and 10.5 ± 0.7 clusters per 7.5×10^4 cells plated, as compared to 112 ± 9 colonies and 143 ± 3 clusters in the presence of 10% WEHI-3B as an optimal exogenous source of CSF. However, pCSF supported significantly more colony formation than control medium (0.3 ± 0.3 colonies, 12 ± 8 clusters; $p < 0.01$, two-tailed Student's *t*-test). Colonies consisted of very small cells of granulocytic morphology by hematoxylin stain.

The murine factor-dependent hemopoietic cell line FDC-P2 proved unresponsive to pCSF when assayed for [³H]TdR uptake after 24, 48, and 72 h in culture. A murine long-term mast cell line showed low levels of responsiveness to pCSF (Y. Yung, Memorial Sloan-Kettering Cancer Center, unpublished observation).

Pluripoietin Enhances Chemotactic Peptide Binding on Mature Granulocytes. Although CSF are defined mainly by their activity on colony-forming hemopoietic progenitor cells, evidence is accumulating that CSF have important biological effects on mature cells of the hemopoietic system like granulocytes, macrophages, or mast cells (reviewed in 16). When pCSF was tested for activity on mature cells, 5,000 U/ml of pCSF acted within minutes to enhance specific binding of the chemotactic peptide fMLP to highly enriched populations of mature human peripheral blood neutrophil granulocytes (Table V). Similar though less pronounced effects were observed at concentrations of pCSF as low as 300 U/ml (data not shown). No significant change in fMLP binding was seen

for 30 min in the absence of pCSF. Although these experiments do not discriminate between increased numbers of receptors or increased affinity for fMLP, the effect of pCSF on specific fMLP binding was highly significant ($p < 0.01$ at 30 min in each experiment of Table V, two-tailed Student's *t*-test). Control experiments excluded bacterial endotoxin contamination of pCSF preparations as a possible cause of enhanced binding of chemotactic peptide. Under our experimental conditions, up to 1 ng/ml of endotoxin (Novopyrexal; Hermal-Chemie, Hamburg, Federal Republic of Germany) did not change fMLP binding to mature granulocytes. In contrast, 1,000 U/ml of pCSF, containing <0.5 ng/ml endotoxin increased fMLP binding more than twofold over that induced by 100 ng/ml endotoxin (data not shown).

Discussion

The present era of cytokine research is characterized by an increasing number of factors becoming available in purified natural or recombinant form. New studies have to compare these well-defined factors and evaluate them for activities originally ascribed to a plethora of cytokines that were operationally defined by the assays used for their study.

We report here on some biological activities of natural pluripotent CSF, purified to homogeneity from CM of the human bladder carcinoma cell line, 5637. As reported earlier (14), pluripoietin (pCSF) supports colony formation in the presence of erythropoietin from normal human multipotential and early erythroid progenitors. This feature distinguishes pluripoietin from the other purified human growth factors, GM-CSF, CSF-1, and EPA, all of which lack this activity partially or completely. Also, pluripoietin differs from human GM-CSF by amino acid sequence analysis (our unpublished observation). In contrast to pluripoietin, CSF-1 appears to be a dimeric molecule (3, 10), and eight neutralizing rabbit antisera to CSF-1 (anti-L cell CSF, generously provided by R. Shadduck, Department of Medicine, Montefiore Hospital, Pittsburgh, PA) did not neutralize pluripoietin in the CFU-GM assay (our unpublished observation). The only murine CSF known to support multipotential colony growth, IL-3, initially appeared to be a possible murine homologue to pluripoietin. However, as will be discussed below, IL-3 lacks the GM-DF activity of pluripoietin.

Experiments reported here suggest that pluripoietin acts on immature progenitor cells supporting their survival, proliferation, and/or differentiation to committed CFU-GM progenitors in suspension culture. This activity of pluripoietin seems to be similar to a granulocyte-promoting activity, δ -GPA, partially purified from human placental CM (34). No such experiments were reported on IL-3, but it seems to have a similar effect on murine stem cells *in vitro* before bone marrow transplantation (35, 36). Murine GM-CSF was reported to support survival *in vitro* and initial proliferative of multipotential and erythroid progenitors (37); similarly, murine G-CSF sustains survival and initial proliferation of CFU-GM and some multipotential progenitors in fetal liver (38). In contrast to pluripoietin, neither murine GM-CSF nor G-CSF are able to sustain continued proliferation of these progenitors resulting in multipotential colony formation.

Like most other CSF, pluripoietin appears to be acting directly on the progenitor cell level, since depletion from the target cell population of CSF-producing

adherent cells and T lymphocytes did not abolish colony formation. Yet pluripoietin also acts on mature hemopoietic cells by enhancing specific binding of the chemotactic peptide fMLP to mature normal granulocytes. This is intriguingly similar to a report (39) that preincubation of polymorphonuclear cells with either purified natural or recombinant human GM-CSF increases superoxide anion production in response to fMLP. There are no published reports on activities of IL-3 on mature granulocytes, but IL-3 is known to act on murine mast cells, inducing histamine synthesis (40) and polychondroitin E sulfate production (41). Other human and murine CSF are thought to induce in mature cells effector functions like antibody-dependent cellular cytotoxicity, plasminogen activator production, tumoricidal and microbicidal activities (reviewed in 16). As previously reported (42), however neither purified pluripoietin nor purified human CSF-1 enhanced H_2O_2 production by mature macrophages in an assay system that optimally detected γ -interferon-induced macrophage activation (42).

A major distinctive feature of pluripoietin is its ability to induce differentiation of the human and murine leukemia cell lines HL-60 and WEHI-3B (D+). In contrast, IL-3 lacks GM-DF activity (16). Murine G-CSF is known to be a potent inducer of leukemic cell differentiation by its ability to induce of phenotypic maturation (26, 27) and clonal extinction (24) in WEHI-3B (D+) cells. Purified murine GM-CSF, a less potent inducer of leukemia differentiation, has failed to completely suppress the progressive proliferation of WEHI-3B (D+) cells since, on serial recloning, leukemic cells maintained in the presence of CSF continued to generate cells capable of forming undifferentiated colonies (43).

In this context, important practical and theoretical implications arise from the finding that pluripoietin may have the potential to inhibit leukemic cell growth by inducing terminal differentiation and at the same time sustain normal hemopoiesis and activate effector functions in mature cells. A human CSF (urinary CSF) has been used already in clinical trials (44). In leukemia, careful consideration will have to be given to the possibility of promoting leukemic cell growth in vivo. In vitro, KG-1 leukemia cell line and CML bone marrow cells were stimulated by purified pluripoietin to increased proliferation in suspension and agar culture. Possibly, clonal assays, including study of secondary plating efficiency (PE-2) in vitro, may provide a means of weighing the combined effect of proliferation and differentiation-inducing stimuli. Studies are under way to determine how many leukemias will be susceptible to the differentiation-inducing potential of pluripoietin.

Recently,² we have succeeded in highly purifying a second CSF from 5637, which we termed pluripoietin- α . Although this CSF also supports multipotential and erythroid colony formation, it is distinguished from pluripoietin by biochemical characteristics and colony morphology in agar. Pluripoietin and pluripoietin- α act synergistically on normal human bone marrow in terms of colony numbers and size (our unpublished observation). Unlike pluripoietin, however, pluripoietin- α is not active on murine bone marrow or WEHI-3B (D+) cells.

Recently, others (45) reported studies on CSF from 5637-CM. They also observed at least two factors in 5637-CM. Although they have not purified these factors, there are some interesting data suggesting that pluripoietin could be a

human homologue to murine G-CSF. Evidence was derived from binding/competition studies of purified murine G-CSF to human and murine normal and leukemic cells. 5637-CM fractionated on phenyl-Sepharose column separated into two peaks of CSF activity, designated CSF- α and CSF- β , in analogy to earlier work (46) on mouse and human CSF. No evidence was provided that separation of the two factors was complete or that activities ascribed to CSF- β are due to only one factor. Purified murine G-CSF and fractions containing human CSF- β competed for receptors on human leukemic promyelocytes and murine myelomonocytic leukemia cells. Also, murine G-CSF and human CSF- β both were potent inducers of differentiation of WEHI-3B (D+), and supported murine normal bone marrow colony formation with a cloning efficiency and morphology similar to that supported by pluripoietin. Therefore, murine G-CSF and partially purified human CSF- β show some intriguing similarities to pluripoietin. As discussed above however, published data (38) indicate that murine G-CSF, unlike pluripoietin, does not support the growth of multipotential colonies and BFU-E. Others (47) have found activities supporting the growth of human multipotential progenitor cells in 5637-CM as well, however, no biochemical characterization of these activities was reported. Clarification of this point will have to await purification of CSF- β and/or sequence data on murine G-CSF.

Another group (48, 49) reported purification of a factor from 5637-CM, using as an assay the induction of receptors for purified murine CSF-1 on murine bone marrow cells. Based on these studies, a factor designated hemopoietin-1 was described in 5637-CM that induced CSF-1 receptors in bone marrow cells from 5-fluorouracil (5-FU)-treated mice within 3–5 d in culture. Since 5-FU treatment depletes CFU-GM progenitor cells and CSF-1-responsive cells, hemopoietin-1 was assumed to work on earlier progenitor cells. Purified hemopoietin-1 had no detectable murine colony-stimulating activity by itself, but it appeared to be a multilineage hemopoietic growth factor capable of synergizing with a CSF other than CSF-1 to support murine multipotential colony growth (49). Because human pluripoietin, in contrast to pluripoietin- α (our unpublished data), has some detectable murine CSF activity, and because of biochemical characteristics, hemopoietin-1 appears to be similar to pluripoietin- α rather than pluripoietin.

It remains for future studies to resolve how many different molecular species of CSF there are. Sequence data available to date indicate no significant degree of inter- or intraspecies homology for human and murine CSF. The availability of purified and cloned growth factors for biological studies is one step in the elucidation of the complex mechanisms involved in regulation of hemopoiesis.

Summary

We studied the biological effects of pluripoietin, a human pluripotent hemopoietic colony-stimulating factor (CSF) purified from the 5637 bladder carcinoma cell line. We found that this human CSF appears to be a unique hemopoietic growth factor, differing from interleukin 3 (IL-3) by virtue of its leukemia differentiating activity in mouse and man, and from mouse granulocyte CSF, which does have differentiation-inducing activity, but lacks pluripoietic activity. In addition, differences from IL-3 were observed in cross-species activity on normal and leukemic cells.

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References

1. Burgess, A. W., and D. Metcalf. 1980. The nature and action of granulocyte-macrophage colony-stimulating factors. *Blood*. 56:947.
2. Metcalf, D. 1982. Regulation of macrophage production. In *Macrophages and natural killer cells*. S. J. Norman and E. Sorkin, editors. Plenum Press, New York. 33-48.
3. Stanley, E. R., and P. M. Heard. 1977. Factors regulating macrophage production and growth: purification and some properties of the colony stimulating factor from medium conditioned by mouse L cells. *J. Biol. Chem.* 252:4305.
4. Burgess, A. W., J. Camarakis, and D. Metcalf. 1977. Purification and properties of colony-stimulating factor from mouse lung-conditioned medium. *J. Biol. Chem.* 252:1998.
5. Nicola, N. A., D. Metcalf, M. Matsumoto, and G. R. Johnson. 1983. Purification of a factor inducing differentiation in murine myelomonocytic leukemia cells. *J. Biol. Chem.* 258:9017.
6. Ihle, J. N., J. Keller, L. Henderson, F. Klein, and E. Palazsynski. 1982. Procedures for the purification of interleukin 3 to homogeneity. *J. Immunol.* 129:2431.
7. Fung, M. C., A. J. Hapel, S. Ymer, D. R. Cohen, R. M. Johnson, H. D. Campbell, and I. G. Young, 1984. Molecular cloning of cDNA for murine interleukin 3. *Nature (Lond.)*. 307:233.
8. Gough, N. M., J. Gough, D. Metcalf, A. Kelso, D. Grail, N. A. Nicola, A. W. Burgess, and A. R. Dunn. 1985. Molecular cloning of cDNA encoding a murine haematopoietic growth regulator, granulocyte-macrophage colony stimulating factor. *Nature (Lond.)* 309:763.
9. Das, S. K., E. R. Stanley, L. J. Guilbert, and L. W. Forman. 1981. Human colony-stimulating factor (CSF-1) radioimmunoassay: resolution of three subclasses of human colony-stimulating factors. *Blood*. 58:630.
10. Das, S. K., and E. R. Stanley. 1982. Structure-function studies of a colony-stimulating factor (CSF-1). *J. Biol. Chem.* 257:13679.
11. Motoyoshi, K., T. Suda, K. Kusumoto, F. Takaku, and Y. Miura. 1982. Granulocyte-macrophage colony-stimulating and binding activities of purified human urinary colony-stimulating factor to murine and human bone marrow cells. *Blood*. 60:1378.
12. Westbrook, C. A., J. E. Gasson, S. E. Gerber, M. E. Selsted, and D. W. Golde. 1984. Purification and characterization of human T-lymphocyte-derived erythroid-potentiating activity. *J. Biol. Chem.* 259:9992.
13. Gasson, J. C., R. H. Weisbart, S. E. Kaufman, S. C. Clark, R. M. Hewick, G. C. Wong, and D. W. Golde. 1984. Purified human granulocyte-macrophage colony-stimulating factor: direct action on neutrophils. *Science (Wash. DC)*. 226:1339.
14. Welte, K., E. Platzer, L. Lu, J. L. Gabilove, E. Levi, R. Mertelsmann, and M. A. S. Moore. 1985. Purification and biochemical characterization of human pluripotent hematopoietic colony-stimulating factor. *Proc. Natl. Acad. Sci. USA*. 82:1526.
15. Watson, J. D., and R. L. Prestidge. 1983. Interleukin 3 and colony-stimulating factors. *Immunol. Today*. 4:278.
16. Nicola, N. A., and M. Vadas. 1984. Hemopoietic colony-stimulating factors. *Immunol. Today*. 5:76.
17. Palacios, R., G. Henson, M. Steinmetz, and J. P. McKearn. 1984. Interleukin 3 supports growth of mouse pre-B cell clones in vitro. *Nature (Lond.)*. 309:126.

18. Koefler, H. P., R. Billing, A. J. Lysis, R. Sparkes, and R. Golde. 1980. An undifferentiated variant derived from the human acute myelogenous leukemia cell line KG-1. *Blood*. 56:265.
19. Potter, G., A. N. Mohamed, N. C. Dracopoli, S. L. B. Groshen, R. N. Shen, and M. A. S. Moore. 1985. The action of 1,25-(OH)₂D₃ in nude mice bearing transplantable human myelogenous leukemic cell lines. *Exp. Hematol. (N.Y.)*. In press.
20. Welte, K., E. Platzer, C. Y. Wang, E. A. Rinnooy Kan, M. A. S. Moore, and R. Mertelsmann. 1983. OKT8 antibody inhibits OKT3-induced IL-2 production and proliferation in OKT8⁺ cells. *J. Immunol.* 131:2356.
21. Platzer, E., B. Y. Rubin, L. Lu, K. Welte, H. E. Broxmeyer, and M. A. S. Moore. 1985. OKT3 monoclonal antibody induces production of colony-stimulating factor(s) for granulocytes and macrophages in cultures of human T lymphocytes and adherent cells. *J. Immunol.* 134:265.
22. Broxmeyer, H. E., L. Lu, E. Platzer, C. Feit, L. Juliano, and B. Y. Rubin. 1983. Comparative analysis of the influences of human gamma, alpha and beta interferons on human multipotential (CFU-GEMM), erythroid (BFU-E), and granulocyte-macrophage (CFU-GM) progenitor cells. *J. Immunol.* 131:1300.
23. Jacobsen, N., H. E. Broxmeyer, E. Grossbard, and M. A. S. Moore. 1979. Colony-forming units in diffusion chambers (CFU-d) and colony-forming units in agar culture (CFU-c) obtained from normal human bone marrow: a possible parent-progeny relationship. *Cell. Tissue Kinet.* 12:213.
24. Metcalf, D. 1980. Clonal extinction of myelomonocytic leukemic cells by serum from mice injected with endotoxin. *Int. J. Cancer.* 25:225.
25. Fibach, E., T. Peled, and E. A. Rachmilewitz. 1982. Modulation of the maturation of human leukemic cells promyelocytes (HL-60) to granulocytes or macrophages. *J. Cell. Physiol.* 113:152.
26. Nicola, N. A., and D. Metcalf. 1981. Biochemical properties of differentiation factors for murine myelomonocytic leukemic cells in organ-conditioned media. Separation from colony-stimulating factors. *J. Cell. Physiol.* 109:253.
27. Cooper, P. C., D. Metcalf, and A. W. Burgess. 1982. Biochemical and functional characterization of mature progeny purified from a myelomonocytic leukemia cell line. *Leuk. Res.* 6:313.
28. Venuta, S., R. Mertelsmann, K. Welte, S. P. Feldman, C. Y. Wang, and M. A. S. Moore. 1983. Production and regulation of interleukin 2 in human lymphoblastic leukemias studied with T cell monoclonal antibodies. *Blood.* 61:781.
29. Harris, P., P. Ralph, P. Litkofski, and M. A. S. Moore. 1985. Distinct activities of gamma interferon, lymphokine, and the cytokine factors inducing terminal differentiation in the human monocytic leukemia cell line U937. *Cancer Res.* 45:9.
30. Koefler, H. P., and D. W. Golde. 1978. Acute myelogenous leukemia: a human cell line responsive to colony-stimulating activity. *Science (Wash. DC)*. 200:1153.
31. Fauser, A. A., and G. W. Loehr. 1982. Recloned colonies positive for T-cell-associated antigens derived from mixed hemopoietic colonies (CFU-GEMM). *Proc. Soc. Exp. Biol. Med.* 170:220.
32. Ogawa, M., P. N. Porter, and T. Nakahata. 1983. Renewal and commitment to differentiation of hemopoietic stem cells (an interpretive review). *Blood.* 61:823.
33. Metcalf, D., and S. Merchav. 1982. Effects of GM-CSF deprivation on precursors of granulocytes and macrophages. *J. Cell. Physiol.* 112:411.
34. Hoang, T., N. N. Iscove, and M. A. S. Moore. 1983. Macromolecules stimulating human granulocytic colony-forming cells, and primitive erythroid progenitors: some apparent non-identities. *Blood.* 61:960.
35. Wagemaker, G., and L. Dorssers. 1984. Properties of purified stem cell activating

- factor (SAF) and its use for selective multiplication of stem cells for bone marrow transplantation in mice. *Exp. Hematol.* 12:454a (Abstr.).
36. Rennick, D. M., F. D. Lee, T. Yokota, K. Arai, H. Cantor, and G. J. Nabel. 1985. A cloned MCGF cDNA encodes a multilineage hematopoietic growth factor: multiple activities of interleukin 3. *J. Immunol.* 134:910.
 37. Metcalf, D., G. R. Johnson, and A. W. Burgess. 1980. Direct stimulation by purified GM-CSF of the proliferation of multipotential and erythroid precursor cells. *Blood.* 55:138.
 38. Metcalf, D., and N. A. Nicola. 1983. Proliferative effects of purified granulocyte colony-stimulating factor (G-CSF) on normal mouse hemopoietic cells. *J. Cell. Physiol.* 116:198.
 39. Weisbart, R. H., D. W. Golde, S. C. Clark, G. C. Wong, and J. C. Gasson. 1985. Human granulocyte macrophage colony-stimulating activity is a neutrophil activator. *Nature (Lond.)*. 314:361.
 40. Ihle, J. N., J. Keller, S. Oroszlan, L. E. Henderson, T. D. Copeland, F. Fitch, M. B. Prystowsky, E. Goldwasser, J. W. Schrader, E. Palazsynski, M. Dy, and B. Lebel. 1983. Biologic properties of homogeneous interleukin. 3. *J. Immunol.* 131:282.
 41. Razin, E., J. N. Ihle, D. Seldin, J. Mencia-Huerta, H. R. Katz, P. A. Leblanc, A. Hein, J. P. Caulfield, K. F. Austen, and R. L. Stevens. 1984. Interleukin 3: a differentiation and growth factor for the mouse mast cell that contains chondroitin sulfate E proteoglycan. *J. Immunol.* 132:1479.
 42. Nathan, C. F., T. J. Prendergast, M. E. Wiebe, E. R. Stanley, E. Platzer, H. G. Remold, K. Welte, B. Y. Rubin, and H. W. Murray. 1984. Activation of human macrophages. *J. Exp. Med.* 160:600.
 43. Metcalf, D. 1979. Clonal analysis of the action of GM-CSF on the proliferation and differentiation of myelomonocytic leukemic cells. *Int. J. Cancer.* 24:616.
 44. Motoyoshi, K., F. Takaku, and Y. Miura. 1983. High serum colony-stimulating activity of leukocytopenic patients after intravenous infusions of human urinary colony-stimulating factor. *Blood.* 62:685.
 45. Nicola, N. A., C. G. Begley, and D. Metcalf. 1985. Identification of the human analogue of a regulator that induces differentiation in murine leukaemic cells. *Nature (Lond.)*. 314:625.
 46. Morstyn, G., N. A. Nicola, and D. Metcalf. 1981. Separate actions of different colony-stimulating factors from human placental conditioned medium on human hemopoietic progenitor cells survival and proliferation. *J. Cell. Physiol.* 109:133.
 47. Myers, C. D., F. E. Katz, G. Joshi, and J. L. Miller. 1984. A cell line secreting factors for CFU-GEMM culture. *Blood.* 64:152.
 48. Bartelmez, S. H., and E. R. Stanley. 1985. Synergism between hemopoietic growth factors (HGFs) detected by their effects on cells bearing receptors for a lineage specific HGF: assay of hemopoietin-1. *J. Cell. Physiol.* 122:370.
 49. Jubinsky, P. T., and E. R. Stanley. 1985. Purification of hemopoietin 1: a multilineage hemopoietic growth factor. *Proc. Natl. Acad. Sci. USA.* 82:2764.